



Research article

Effect of sulfur dioxide on ROS production, gene expression and antioxidant enzyme activity in *Arabidopsis* plantsLihong Li^{a,b}, Huilan Yi^{a,*}^a School of Life Science, Shanxi University, Taiyuan 030006, China^b Fisheries Research Institute of Shanxi Province, Taiyuan 030006, China

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ABSTRACT

Sulfur dioxide (SO₂) is one of the most common and harmful air pollutants. To analyze antioxidant response of plants to SO₂ stress, we investigated the reactive oxygen species (ROS) levels, transcript alterations and antioxidant enzyme activities in *Arabidopsis thaliana* (Col-0) exposed to 0, 2.5, 10 and 30 mg m⁻³ of SO₂. The results showed that both superoxide radical (O₂^{-•}) generation rate and hydrogen peroxide (H₂O₂) content increased in SO₂-treated *Arabidopsis* shoots. GeneChip and RT-PCR analysis revealed that transcript levels of peroxidase (POD), glutathione peroxidase (GPX) and superoxide dismutase (SOD) genes enhanced after exposure to 30 mg m⁻³ SO₂ for 72 h. The content of glutathione and activities of SOD, POD and GPX increased significantly during 72 h of SO₂ exposure. However, catalases (CAT) activity changed slightly under SO₂ stress. Furthermore, the results of in-gel enzyme assays indicated that SOD (FeSOD and Cu/ZnSOD) and POD isoforms increased after exposure to SO₂ for 72 h, whereas two CAT isoforms (CAT2 and CAT3) declined. Malondialdehyde content kept at a low level within 72 h of SO₂ exposure, but increased significantly after exposure to 30 mg m⁻³ SO₂ for 120 h along with decrease in the level of ROS and activities of SOD and GPX. Our results indicated that increased ROS may act as a signal to induce defense response to SO₂ stress. Antioxidant status plays an important role in plant protection against SO₂-caused oxidative stress, though the defense capacity cannot sufficiently alleviate oxidative damage occurring under prolonged exposure to higher concentrations of SO₂.

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1. Introduction

Sulfur dioxide (SO₂) is one of the most common and harmful air pollutants. The phytotoxicity of SO₂ strongly depends on its concentration and exposure duration, and is also influenced by the sulfur status of plants [1]. Low doses of SO₂ may be harmless or even beneficial to plants since sulfur is an essential macronutrient for plants [2,3]. Sulfur is a structural component of amino acids, proteins, vitamins and chlorophyll [1,4]. Sulfur enhances the development of nodules and nitrogen fixation by legumes and also affects carbohydrate metabolism. However, exposure to high doses of SO₂ can induce leaf chlorosis and necrosis, growth inhibition, and plant death [5,6]. The concentration of SO₂ in the atmosphere has increased in many areas of the world during the past few decades. It

is estimated that parts of global plants will be exposed to potentially phytotoxic levels of SO₂ and this concern has prompted widespread attempts to evaluate plant defense responses [7].

The toxicity of SO₂ is derived from the toxic molecular species sulfite (SO₃²⁻) and bisulfite (HSO₃⁻) generated after SO₂ dissolved in cellular cytoplasm [8,9]. Furthermore, detoxification reaction of (SO₃²⁻) to sulfate (SO₄²⁻) in plants leads to the enhancement of reactive oxygen species (ROS), such as superoxide radical (O₂^{-•}), hydrogen peroxide (H₂O₂) and hydroxyl radical (OH[•]) [10]. Increased ROS can attack biomacromolecules and result in oxidative damage to nucleic acids, proteins and lipids [11–13]. However, plants can scavenge excess ROS by invoking the antioxidant defense system to avoid oxidative damage [14,15]. Many studies have showed that some antioxidant enzymes are involved in plant response to SO₂ stress [16,17]. The induction of antioxidant enzymes is thought to be a protective reaction of plants against SO₂ stress, but the exact defense mechanism is not clear.

In our previous study we have identified a dose- and time-dependent change in morphological features and physiological indexes in response to SO₂ in *Arabidopsis* plants [18]. In the present study, we selected the exposure condition to conduct a systematic

Abbreviations: CAT, catalases; GPX, glutathione peroxidase; GSH, glutathione; H₂O₂, hydrogen peroxide; MDA, malondialdehyde; O₂^{-•}, superoxide radical; POD, peroxidase; ROS, reactive oxygen species; SO₂, sulfur dioxide; SOD, superoxide dismutase.

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investigation of alterations in ROS production, gene expression and antioxidant enzymes activities in *Arabidopsis* plants exposed to SO₂. Our research provided a particular insight into the capacity of SO₂ to induce cellular ROS and antioxidant response in plant cells, contributing to the mechanisms of plant adaptation to SO₂ stress.

2. Results

2.1. SO₂-induced ROS generation

Exposure to SO₂ caused an increase in ROS generation in *Arabidopsis* shoot cells. The O₂^{•−} generation rate and H₂O₂ content enhanced with increasing SO₂ concentration and prolonged exposure duration within 72 h of exposure (Fig. 1A and B). After exposure to 30 mg m^{−3} SO₂ for 72 h, O₂^{•−} generation rate and H₂O₂ content increased by about 90% ($P < 0.01$) and 30% ($P < 0.05$), respectively. However, the O₂^{•−} generation rate and H₂O₂ content reduced after exposure to SO₂ for a long term (120 h).

2.2. SO₂-induced antioxidant defense response

We analyzed the temporal evolution of stress responses in *Arabidopsis* plants exposed to 30 mg m^{−3} SO₂ for 72 h. This treatment is sufficient to evoke physiological and transcriptional changes, but does not induce tissue damage, as indicated by absence of visible lesions and low levels of lipid peroxidation.

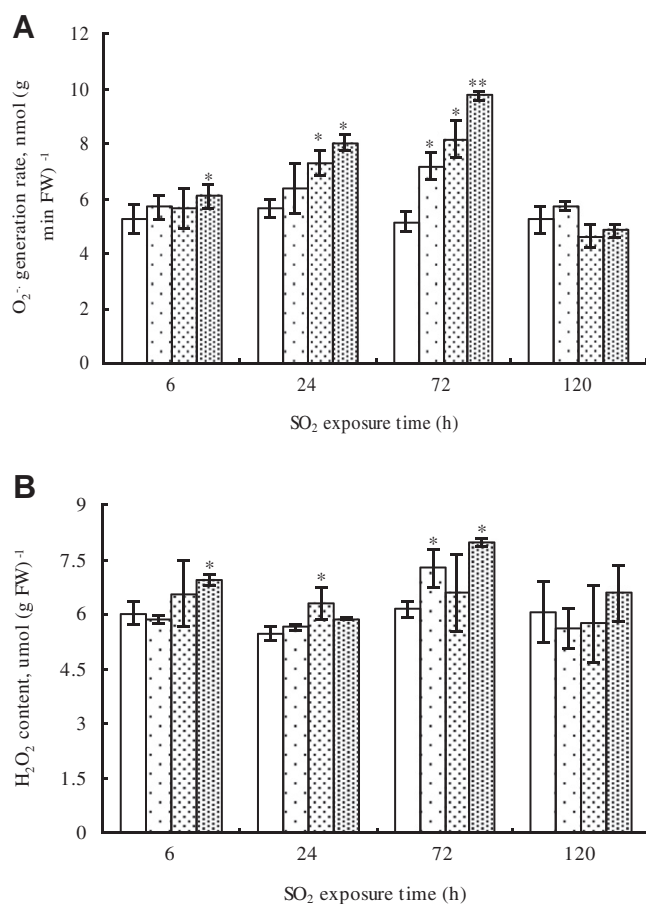


Fig. 1. Effect of SO₂ on O₂^{•−} generation rate (A) and H₂O₂ content (B) in *Arabidopsis* shoots treated with 0 (□), 2.5 (▤), 10 (▥) and 30 (▧) mg m^{−3} SO₂ for 120 h. The results are expressed as means ± SE ($n = 3$). * $P < 0.05$, ** $P < 0.01$.

Transcriptional profiling revealed that 2780 genes were differentially expressed ($R \geq 1$ -fold change) in *Arabidopsis* plants exposed to 30 mg m^{−3} SO₂ for 72 h. Functional annotation clustering analysis revealed that these differentially expressed genes were mainly involved in binding, metabolism, transcription regulation, signal transduction, molecular structure, and transport, and so on. A number of defense-related genes encoding antioxidant defense enzymes, cytochrome P450, heat shock proteins (Hsps) and pathogenesis-related (PR) proteins were significantly up-regulated in SO₂-treated *Arabidopsis* shoots. Ten antioxidant genes were up-regulated in *Arabidopsis* shoot cells during SO₂ exposure: five peroxidase (POD) genes, three glutathione peroxidase (GPX) genes and two Cu/Zn superoxide dismutases (CSD1 and CSD2) genes (Table 1).

The expression pattern of four differentially expressed antioxidant genes selected from microarray data was validated using semi-quantitative RT-PCR: Cu/Zn superoxide dismutase (At1g08830, CSD1; At2g28190, CSD2), glutathione peroxidase (At4g31870, GPX7) and peroxidase (At4g33420, POD). RT-PCR results indicated that these genes were strongly up-regulated in SO₂-treated samples (Fig. 2), consistent with the results from microarray analysis.

To further understand the response of antioxidant system to SO₂ exposure, we analyzed the content of glutathione (GSH) and activities of superoxide dismutases (SOD), catalase (CAT), POD and GPX in SO₂-treated *Arabidopsis* plants. Our results showed that GSH content increased significantly after exposure to SO₂ for 6 h and then the increment of GSH content declined with prolonged exposure duration. After exposure to 2.5, 10 and 30 mg m^{−3} SO₂ for 120 h, GSH contents increased by 7%, 1% and 13%, respectively (Fig. 3).

The activities of SOD and GPX enhanced in dose- and time-dependent manners during 72 h of SO₂ exposure. After exposure to 30 mg m^{−3} SO₂ for 72 h, the activities of SOD and GPX increased by about 45% ($P < 0.01$) and 119% ($P < 0.01$), respectively. However, the activities of SOD and GPX decreased in *Arabidopsis* shoots exposed to SO₂ for 120 h (Fig. 4A and B).

POD activity increased significantly in *Arabidopsis* shoots exposed to SO₂ for 6 h and maintained higher levels during 120 h of SO₂ exposure. After exposure to 30 mg m^{−3} SO₂ for 120 h, POD activity increased by up to 146% ($P < 0.01$) (Fig. 4C).

CAT activity changed slightly in *Arabidopsis* shoots during 120 h of SO₂ exposure. The CAT activity reduced with prolonged exposure duration, but the differences were not statistically significant (Fig. 4D).

2.3. SO₂-induced changes of antioxidant enzymes isoenzyme zymogram

Exposure to SO₂ induced changes in the intensities of SOD, POD and CAT isoenzyme bands. Seven well-resolved bands of SOD

Table 1
List of selected antioxidant genes up-regulated in response to SO₂ stress ($R \geq 1$).

ID	Gene name	Fold change
At1g08830	Copper/zinc superoxide dismutase (CSD1)	1.4
At2g28190	Copper/zinc superoxide dismutase (CSD2)	1.5
At4g31870	Glutathione peroxidase (GPX7)	1.6
At1g63460	Glutathione peroxidase (GPX8)	1.4
At2g31570	Glutathione peroxidase (GPX2)	1.3
At4g33870	Peroxidase (POD)	2
At5g05340	Peroxidase (POD)	1.9
At4g17690	Peroxidase (POD)	1.7
At4g37530	Peroxidase (POD)	1.5
At4g33420	Peroxidase (POD)	1.5

isoforms were detected in *Arabidopsis* shoot cells (Fig. 5A). Band 1 and 2 were identified as MnSOD and FeSOD, respectively, and the other five bands were identified as Cu/ZnSODs. SO₂ increased FeSOD (SOD-2) isoform, whereas had no significant effect on MnSOD (SOD-1) isoform. All Cu/ZnSOD (band 3–7) isoforms enhanced after exposure to 2.5 mg m⁻³ SO₂ for 72 h. However, apparent differences in intensity of several Cu/ZnSOD isoforms were observed after exposure to 30 mg m⁻³ SO₂ for 72 h.

Two CAT isoforms, CAT2 and CAT3, were detected in *Arabidopsis* shoot cells (Fig. 5B). CAT2 and CAT3 isoforms changed slightly after exposure to 2.5 and 10 mg m⁻³ SO₂, but both CAT isoforms decreased after exposure to 30 mg m⁻³ SO₂.

Three POD isoform bands were detected in *Arabidopsis* shoot cells (Fig. 5C). The intensity of band 1 and 2 increased significantly after exposure to 30 mg m⁻³ SO₂, and band 3 increased after exposure to 2.5 mg m⁻³ SO₂.

2.4. SO₂-induced lipid peroxidation

The level of lipid peroxidation in SO₂-treated *Arabidopsis* plants was measured as the content of malondialdehyde (MDA). Within 72 h of SO₂ exposure, MDA content was virtually unchanged in *Arabidopsis* shoots. However, MDA content increased significantly ($P < 0.05$) after exposure to 30 mg m⁻³ SO₂ for 120 h (Fig. 6), indicating that prolonged exposure to higher concentrations of SO₂ caused oxidative damage in plant cells.

3. Discussion

In plant cells, ROS are unavoidable by-products of aerobic metabolism. Under normal growth conditions, amounts of ROS are modest and cells experience only mild oxidative stress, whereas many stresses enhance ROS production [11,14]. The results of our present study clearly showed that SO₂ triggered rapid increase in O₂⁻ generation rate and H₂O₂ content in *Arabidopsis* shoots. The enhanced production of ROS under stress can pose a threat to cells, but it is also thought that ROS serve as signal molecules to activate the stress responses and defense pathways [15,19,20]. Thus, SO₂-induced ROS can be viewed as cellular indicators of stress and as secondary messengers involved in the stress-response signal transduction pathway.

In this study, SO₂ enhanced gene expression and enzymatic activities of several antioxidant enzymes including SOD, POD and GPX associated with increase in ROS generation, suggesting an induction of antioxidant defenses regulated by ROS mediated signaling pathway. The genome-wide transcript analysis revealed that many defense-related genes encoding antioxidant defense enzymes, Hsps, cytochrome P450 and PR proteins were up-regulated in *Arabidopsis* shoot cells after exposure to 30 mg m⁻³ SO₂ for 72 h, suggesting that plants can develop adaptive metabolic means of tolerance to SO₂ pollution. Increasing antioxidant levels in *Arabidopsis* cells through modification of gene expression and adaptive metabolism protect plants against SO₂-caused oxidative damage. It is possible that the anti-oxidative capacity and the SO₂-induced response in *Arabidopsis* contribute to an efficient protection against SO₂ stress.

SOD catalyzes the dismutation reaction of O₂⁻ into H₂O₂ and O₂, and can be divided into three classes according to their metal co-factors: Cu/ZnSOD, MnSOD, and FeSOD [21]. FeSODs are located in chloroplast, MnSODs in mitochondrion and peroxisome, and Cu/ZnSODs in chloroplast and cytosol, and possibly the extracellular space. Salt stress has been shown to preferentially induce MnSOD [22], but both UV-B and O₃ have been reported to increase Cu/ZnSOD in *Arabidopsis* [16,23]. In the present study, SO₂ increased SOD activity as well as FeSOD and Cu/ZnSOD isoforms, but had no marked effect on MnSOD, demonstrating that SO₂-induced increase in SOD activity was mainly due to the enhancement of FeSOD and Cu/ZnSOD. Also transcript abundance of *CSD1* and *CSD2* was up-regulated in *Arabidopsis* shoots. The difference in response of MnSOD, Cu/ZnSOD and FeSOD in plants might be due to a combination of the influence of their disparate subcellular locations and upstream sequences in the genome [24].

CAT and POD are the primary H₂O₂-scavenging enzymes in plant cells. In this study, SO₂ pronouncedly increased POD activity, whereas had little effect on CAT activity. In consistent with the alteration of enzyme activities, five POD genes were found to be up-regulated in *Arabidopsis* shoot exposed to SO₂. CAT is a high capacity but low affinity enzyme for H₂O₂, whereas POD has a high affinity for H₂O₂ [15]. Thus, POD, but not CAT, was the most efficient scavenging enzyme to decrease the cellular levels of H₂O₂ in plant cells under SO₂ stress. Moreover, the increase of POD activity can contribute to the resistance enhancement since POD participates in many other cell processes involved in plant defense reaction [25]. POD can initiate cell-wall toughening events such as phenolic

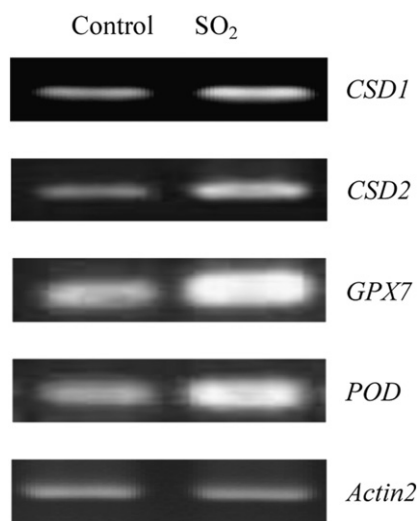


Fig. 2. RT-PCR analysis of four differentially expressed antioxidant genes selected from microarray data. RNA was extracted from the untreated control or SO₂-treated *Arabidopsis* plants.

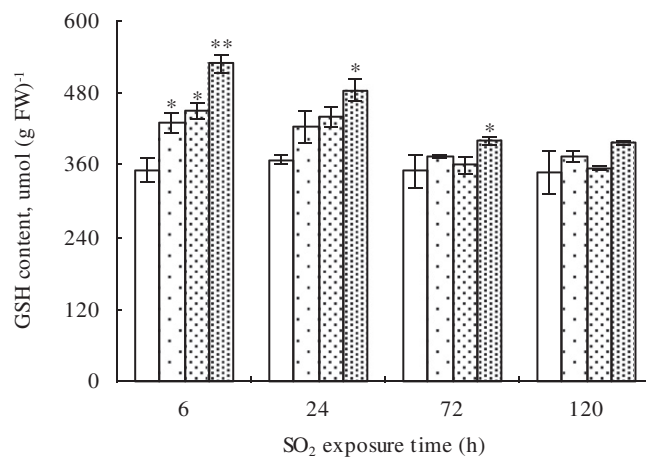


Fig. 3. Effect of SO₂ on GSH content in *Arabidopsis* shoots treated with 0 (□), 2.5 (▒), 10 (■) and 30 (▨) mg m⁻³ SO₂ for 120 h. The results are expressed as means ± SE ($n = 3$). * $P < 0.05$, ** $P < 0.01$.

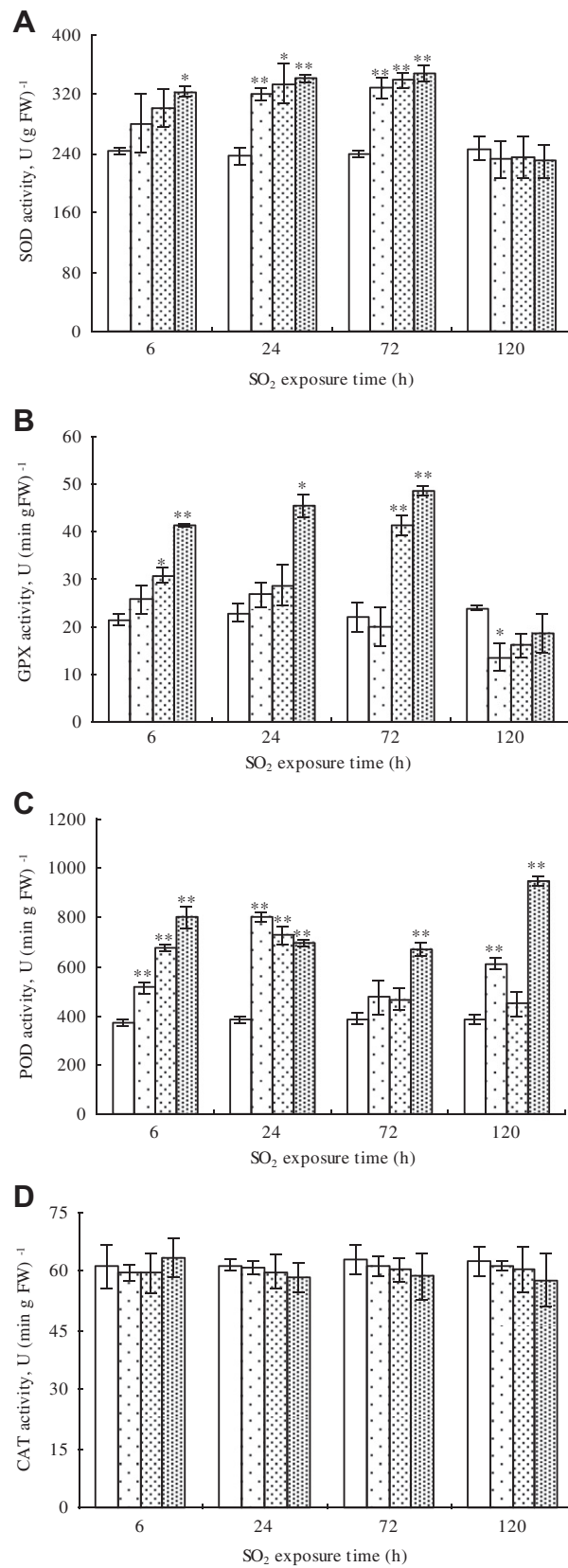


Fig. 4. Effect of SO₂ on the activities of SOD (A), GPX (B), POD (C) and CAT (D) in *Arabidopsis* shoots treated with 0 (□), 2.5 (▨), 10 (▩) and 30 (■) mg m⁻³ SO₂ for 120 h. The results are expressed as means ± SE (*n* = 3). **P* < 0.05, ***P* < 0.01.

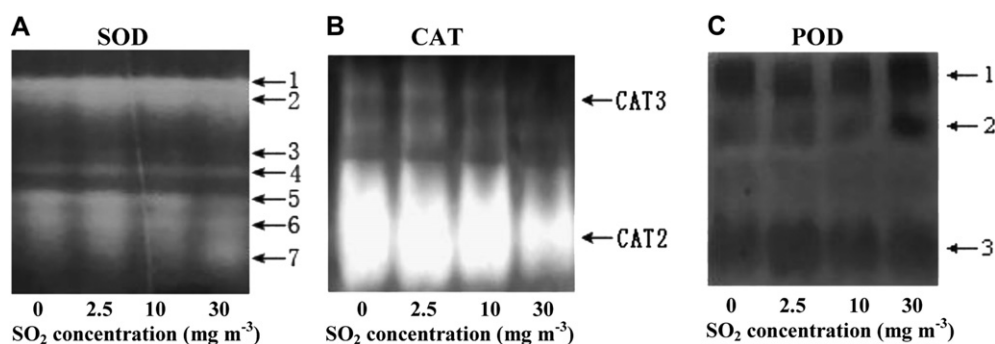


Fig. 5. Isozyme zymogram of SOD (A), CAT (B) and POD (C) in *Arabidopsis* shoots treated with 0, 2.5, 10 and 30 mg m⁻³ SO₂ for 72 h. Samples applied to the gels contained 75 µg of protein.

cross-linking and lignification, which can strengthen leaf and stem tissues against potential damage [26]. The up-regulated expression of POD and other genes involved in the phenylpropanoid pathway to synthesize protective substances also demonstrated the defense effects of the modification of secondary metabolism in SO₂-fumigated plants [27]. The roles that POD can play in cell wall toughening and in the production of secondary metabolites and its simultaneous oxidant and antioxidant capabilities make it an important factor in the integrated defense response of plants to SO₂ stress.

GSH is an important antioxidant, which not only plays a central role in the processes of redox buffering but also acts as a co-substrate for GPX [28]. GPX catalyzes the reduction of H₂O₂ and lipid peroxides using GSH as electron donor [29]. SO₂-induced increase in GSH content and GPX activity can scavenge H₂O₂ and protect the cell membrane against lipid peroxidation. Cysteine, serving as a precursor for GSH synthesis, increased significantly under SO₂ stress [3,8], might contribute to the increase in GSH level and then to the enhancement of chemical resistance.

MDA, the product of lipid peroxidation, has been regarded as an indicator of oxidative damage at cellular level [30]. In this study, SO₂ caused an increase in the generation of O₂⁻ and H₂O₂, but had no marked effect on MDA content in *Arabidopsis* shoots within 72 h of SO₂ exposure, due to the increase of antioxidants contents and antioxidant enzymes activities. However, MDA content increased in *Arabidopsis* shoots after exposure to 30 mg m⁻³ SO₂ for 120 h, along with the decrease in SOD and GPX activities. These results indicated that SO₂ induced an antioxidant response which provided an efficient protection against oxidative damage within 72 h of SO₂ exposure. However, long term exposure to higher concentration of SO₂ caused excess ROS in plant cells leading to cell damage and, in this case, MDA content increased, many enzymes activities such as SOD and GPX declined, and then the cell structure and function might be destroyed.

In conclusion, this is a systematic study which provided a particular insight into the changes in ROS production and antioxidant status in *Arabidopsis* plants under SO₂ stress, contributing to the mechanism of plant adaptation. Our studies suggest that SO₂ exposure caused high levels of ROS and an increased antioxidant defense rapidly. SO₂-induced ROS, such as O₂⁻ and H₂O₂, are able to stimulate antioxidant enzyme gene expression and promote nonenzymatic antioxidant compounds increase, mediating plant adaptation to stress by strengthening antioxidant defense capability. However, increased level of lipid peroxidation product MDA occurred in *Arabidopsis* shoots exposed to SO₂ for a long term (120 h), demonstrating that the antioxidant defense system is no longer capable to destroy free radicals. Antioxidant status may play a critical role in the defense against SO₂ stress.

4. Materials and methods

4.1. Plant materials and SO₂ treatments

Plants of *Arabidopsis thaliana* (L.) ecotype Columbia (Col-0) were grown in a controlled growth chamber at 22 ± 1 °C with a 16 h photoperiod per day, 70% relative humidity and a photosynthetic photon flux density of 140 µmol m⁻² s⁻¹.

Four-week-old plants were exposed to 2.5, 10, 30 mg m⁻³ SO₂, respectively, or to filtered pollutant-free air (control) for up to 120 h in fumigation chambers. The gas was released through a tube from a cylinder and continuously sampled and measured by paraosaniline hydrochloride spectrophotometry to monitor SO₂ concentrations [31]. Shoots were harvested in the middle of the photoperiod after 6, 24, 72 and 120 h of exposure.

4.2. Determination of O₂⁻ generation rate and H₂O₂ content

O₂⁻ generation rate was determined according to the method of Elstner and Heupel [32]. Shoots were homogenized in ice-cold 65 mM sodium phosphate buffer (pH 7.8) and centrifuged at 12,000 × g for 15 min at 4 °C. Supernatants were collected and analyzed for O₂⁻ generation rate. The reaction mixture contained 0.5 mL supernatant, 1.0 mL of 65 mM sodium phosphate buffer (pH 7.8), 0.1 mL of 7.5 mM xanthine, 0.1 mL of 10 mM hydroxylammonium chloride and 0.3 mL xanthine oxidase, and incubated at 25 °C for 20 min. Then 0.5 mL of 19 mM sulfanilic acid and 0.5 mL of 1.0% α-naphthylamine were added to 0.5 mL of above reaction

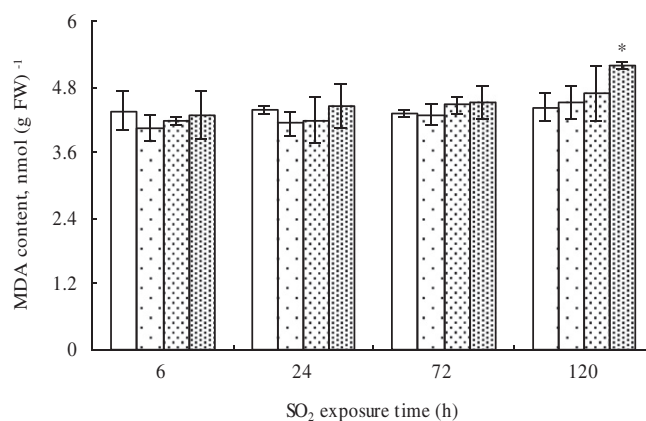


Fig. 6. Effect of SO₂ on MDA content in *Arabidopsis* shoots treated with 0(□), 2.5(▤), 10(▥) and 30(▧) mg m⁻³ SO₂ for 120 h. The results are expressed as means ± SE (n = 3). *P < 0.05.

mixture, and incubated at 25 °C for 20 min. The absorbance was measured at 530 nm against blanks which had been prepared similarly without supernatant.

H₂O₂ content was determined according to the method of Patterson et al. [33]. Shoots were homogenized in ice-cold acetone and centrifuged at 3000 × g for 10 min. Supernatants were collected and analyzed for H₂O₂ content. The reaction mixture contained 0.4 mL supernatant, 0.1 mL of 20% titanium tetrachloride (TiCl₄) and 0.2 mL strong ammonia to give a Ti–H₂O₂ complex (precipitated). The precipitate was dissolved in 3 mL H₂SO₄ and the absorbance was measured at 415 nm against blanks which had been prepared similarly without supernatant.

4.3. RNA isolation and GeneChip hybridization assay

Total RNA from shoots of control and SO₂-treated plants (30 mg m⁻³ SO₂) were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentrations of RNA were measured spectrophotometrically at 260 nm. The 260/280 nm ratio was determined for assessing the purity of prepared RNA samples. The integrity of prepared RNA was examined by electrophoresis in 1.5% agarose gel.

The microarray experiments were performed with the help of National Engineering Center for Biochip at Shanghai (China) according to the Affymetrix GeneChip Expression Analysis Technical Manual. Briefly, labeled cRNAs were fragmented and hybridized overnight (~16 h, 45 °C) to an Affymetrix ATH1 *Arabidopsis* GeneChip microarray containing 22,810 probe sets. Expression analysis in control and SO₂-treated *Arabidopsis* cells was performed using GeneChip Operating Software (GCOS, Affymetrix). A two-tailed paired *t*-test analysis was performed to identify the genes whose expression levels changed significantly (*P*-value < 0.01) after SO₂ treatment. Only the genes with *P*-value < 0.01 were further analyzed and reported. The differential level was represented by a log₂ intensity ratio. When this value equals 1 (or -1), there is 2-fold increase (or decrease) in gene expression.

4.4. Semi-quantitative RT-PCR analysis

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Primers for four selected differentially expressed antioxidant genes are listed in Table 2. *Actin 2* gene was used as an internal control to normalize the expression of target genes. PCR products were subjected to electrophoresis on 1% agarose gel followed by ethidium bromide staining for visualization.

4.5. Determination of GSH content

GSH content was determined according to the method of Jollow et al. [34]. Shoots were homogenized in ice-cold 3% trichloroacetic acid (TCA) and centrifuged at 3000 × g for 10 min. Supernatants were collected and analyzed for GSH content. The reaction mixture contained supernatant, 0.1 M potassium phosphate buffer (pH 7.4)

and 100 mM 5,5-dithiobis (2-nitrobenzoic) acid (DTNB). The absorbance was measured immediately at 412 nm after yellow color developed. GSH content was determined by comparing the absorbance with that of a standard curve made with known amounts of GSH.

4.6. Assay of antioxidant enzymes activities

The activities of SOD, POD, CAT and GPX were measured. Shoots were homogenized in ice-cold 50 mM sodium phosphate buffer (pH 7.8) containing 2 mM ethylenediamine-tetra acetic acid (EDTA) and 1% (w/v) polyvinyl-polypyrrolidone (PVP). After centrifugation at 12,000 × g for 20 min at 4 °C, the supernatants were analyzed for enzyme activities.

SOD (EC 1.15.1.1) activity was determined according to the method of Beauchamp and Fridovich [35]. The reaction mixture contained enzyme extract, 50 mM sodium phosphate buffer (pH 7.8), 10 μM EDTA, 75 μM nitroblue tetrazolium (NBT), 13 mM methionine and 2 μM riboflavin. The mixtures were exposed to white fluorescent illumination for 20 min and the absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme inhibiting NBT reduction by 50%.

POD (EC 1.11.1.7) activity was determined according to the method of Polle et al. [26]. The reaction mixture contained enzyme extract, 100 mM potassium phosphate buffer (pH 6.0), 16 mM guaiacol and 10 μL of 30% H₂O₂. The reaction was initiated by adding enzyme extract and the increase in absorbance was measured at 470 nm. One unit of POD activity was defined as the amount of enzyme causing a change of 0.01 in absorbance per minute.

CAT (EC 1.11.1.6) activity was determined according to the method of Piero et al. [36]. The reaction mixture contained enzyme extract, 100 mM potassium phosphate buffer (pH 7.0) and 10 μL of 30% H₂O₂. The reaction was initiated by adding H₂O₂ and the absorbance was measured at 240 nm. One unit of CAT was defined as the amount of enzyme catalyzing the decomposition of 1 μmol of H₂O₂ per minute.

GPX (EC 1.11.1.9) activity was determined according to the method of Lawrence and Burk [37]. The reaction mixture contained enzyme extract, 0.2 mM NADPH, 1 mM sodium azide (pH 7.0), 1 mM GSH, 1 U glutathione reductase and 2 mM H₂O₂. The reaction was initiated by adding H₂O₂ and the absorbance was measured at 340 nm. One unit of GPX activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADPH per minute.

4.7. Determination of MDA content

MDA content was determined according to the method of Draper and Hadley [38]. Shoots were homogenized in 5% (w/v) trichloroacetic acid (TCA) and centrifuged at 3000 × g for 10 min. Supernatants were collected and reacted with an equal volume of 0.67% (w/v) thiobarbituric acid (TBA) in a boiling water bath for 30 min. After cooling, the mixture was centrifuged at 3000 × g for 10 min. The absorbance of supernatant was measured at 532 nm

Table 2
Primer sequences for RT-PCR reactions.

Gene	Forward primer	Reverse primer
CSD1	5'-AGACGAAGCAAAAACATTTCAGAGA-3'	5'-GGCCAGAACTGTTCCTC-3'
CSD2	5'-ATGGCTGCCACCAACACAATCC-3'	5'-TTAGACGGCGTCAAGCCAATC-3'
GPX7	5'-TTCGCTGCAATCCGTCTCC-3'	5'-ACGTTAACGATCAACAAAGG-3'
POD	5'-ATGACTTACTACATGATGAGCTGTCC-3'	5'-CAGTGTGTCTTTCGTTGAATCTAG-3'
Actin 2	5'-TTCCTCATGCCATCTCTCGTCTT-3'	5'-CAGCGATACCTGAGAACATAGTGG-3'

and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm and 450 nm.

4.8. Assay of in-gel enzyme

Shoots were homogenized in ice-cold 9 mM Tris–HCl (pH 6.8) and 13.6% (v/v) glycerol. After centrifugation at $12,000 \times g$ for 5 min at 4 °C, the supernatants were analyzed for isoenzymes. Soluble protein content was determined according to the method of Bradford [39] with bovine serum albumin (BSA) as the standard. Samples containing equal amounts of soluble protein were subjected to non-denaturing native discontinuous polyacrylamide gel electrophoresis (PAGE) without sodium dodecyl sulfate (SDS).

In-gel assay for SOD isoenzymes was performed using 12% separation gel and 4% stacking gel at 4 °C. After electrophoresis, the gels were incubated in 2.5 mM NBT for 20 min, followed by incubating in 2.8 mM riboflavin for 15 min in the dark. Then, the gels were placed in distilled water and exposed to white fluorescent illumination for 10–15 min. SOD isoenzymes were visualized as colorless bands on the purple background [20]. Different SOD isoenzymes were identified using two inhibitors, 5 mM H_2O_2 or 2 mM KCN. KCN inhibited Cu/Zn-SOD activity and H_2O_2 inhibited Cu/Zn-SOD and FeSOD activities. However, Mn-SOD activity is resistant to both inhibitors [40].

In-gel assay for CAT isoenzymes was performed using 7.5% separation and 4% stacking gels at 4 °C. After electrophoresis, the gels were incubated in 0.01% H_2O_2 solution for 5 min, followed by washing twice in water and incubating in solution of 1% (w/v) $K_3[Fe(CN)_6]$ and 1% (w/v) $FeCl_3$ for 5 min. CAT isoenzymes were visualized as colorless bands on the blue background [41].

In-gel assay for POD isoenzymes was performed using 7.5% separation and 4% stacking gels at 4 °C. After electrophoresis, the gels were incubated in sodium acetate buffer (pH 4.5) containing 2 mM benzidine. The reaction was initiated by adding 3 mM H_2O_2 and incubated at room temperature till brown bands appeared [23].

4.9. Statistical analysis

Each experiment was replicated in triplicate. All results formatted as bar graphs are presented as means \pm SE (standard error). The statistical significance was evaluated by a one way ANOVA followed by Dunnett's test.

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